THE JUVENILE HORMONE. VI. EFFECTS OF THE "PAPER FACTOR" ON THE GROWTH AND METAMORPHOSIS OF THE BUG, PYRRHOCORIS APTERUS [1]

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We have already described the presence in American paper products of a highly active analogue of the juvenile hormone of *Pyrrhocoris apterus* (Sláma and Williams, 1965, 1966). Thus, when reared in contact with "active paper," *Pyrrhocoris* larvae undergo one or more supernumerary larval moults and finally die without completing metamorphosis or attaining sexual maturity. This same result was provoked when 1 μg of partially purified "paper factor" (PF) was applied to larvae at the outset of the normal, final (fifth) larval instar (Sláma and Williams, 1966).

Our present purpose is to describe in further detail the effects of PF on the growth, moulting, and metamorphosis of *Pyrrhocoris apterus*.

Materials and Methods

1. *Culture of Pyrrhocoris*

All cultures were reared at 25 ± 1° C in an incubator equipped with three 15-watt fluorescent lamps programmed for a daily illumination of 17 hours (Williams and Adkisson, 1964). Distilled water was placed in the bottom of the incubator to maintain a moist atmosphere.

The breeding stocks of adult bugs were reared in round glass jars (9 cm O.D. × 9.4 cm) containing: linden seed (*Tilia cordata*), a pleated rectangle of Whatman's No. 1 filter paper, and a cotton-plugged vial of tap-water; the latter was replaced every two or three days. The jars were capped with cotton netting which was held in place with rubber bands.

At daily intervals, the cultures were inverted to filter the fertilized eggs through the cotton netting. Each group of eggs was placed on a 9-cm disc of Whatman's No. 1 filter paper in a 10-cm Petri dish. As soon as the eggs hatched, linden seed and a vial of water were put in each dish, the latter being replaced every two or three days. The larvae were reared to specific stages and then used in the experiments noted below.

2. *Extraction of PF*

Crude PF was extracted from paper towels (Scott Paper Co., Brand 150) by soaking them in absolute methanol. The solvent was removed on a rotary

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evaporator, and the resulting brown sludge was concentrated by extraction into petroleum ether (b.p. 30–60° C). The solution was filtered and chromatographed on a column of silicic acid, as previously described (Sláma and Williams, 1966). The overall purification achieved by these maneuvers was about four-fold.

Weighed samples of the extract were dissolved in acetone or methanol and dispensed with macro- or micropipettes.

3. *Treatment of Pyrrhocoris with PF*

Groups of larvae, at specific stages of development, were placed in 10-cm Petri dishes, along with linden seed and a vial of water, and exposed to PF in one of three ways: (1) a folded rectangle was cut from Scott towelling and placed

TABLE 1

Scoring system for evaluating the effects of juvenile hormone and mimetic substances on fifth instar Pyrrhocoris larvae

Score	5th instar moult into:
0	Normal adults.
I	Slightly affected adults; forewings shortened and deformed with larval cuticle at their tips; destruction of hindwings incomplete; abdominal sternum covered with black adult-type cuticle.
II	Adultoids with forewings of larval shape and proportions; hindwing lobes fully preserved; abdomen with black adult-type cuticle and patches of transparent larval-type cuticle through which red pigmented epidermis may be seen.
III	Adultoids with larval wings (as above); about half of abdomen covered by transparent larval-type cuticle through which red pigmented epidermis may be seen.
IV	Nearly perfect 6th instar larvae, except for black patches of adult cuticle scattered over abdominal sternites.
V	Perfect 6th instar larvae.

in the dish; (2) a 9-cm disc of Whatman's No. 1 filter paper, plus a pleated rectangle cut from the same material, were impregnated with an acetone solution containing a known amount of PF and placed in the dish; or (3) by means of a microsyringe, a measured droplet of PF solution was placed on the abdominal tergum of individual CO_2-anesthetized insects.

RESULTS

1. *Quantitative aspects of the Pyrrhocoris assay*

Exposure of early fifth instar larvae to PF duplicated all the effects previously achieved by the implantation of active corpora allata (Sláma, 1962, 1964). In each case the presence of juvenile hormone activity was signaled by a failure of the larvae to transform into normal adults.

In practice, it was possible to subdivide the juvenile hormone reactions into six intensities, ranging from 0 (metamorphosis into normal adults) to V (moulting to sixth stage larvae with full and complete preservation of larval characters). Table I summarizes the external characters used in this scoring system.

2. *Normal development of Pyrrhocoris*

The duration of a particular instar is ordinarily considered to be the elapsed time between successive ecdyses. When *Pyrrhocoris* larvae are cultured at 25° C

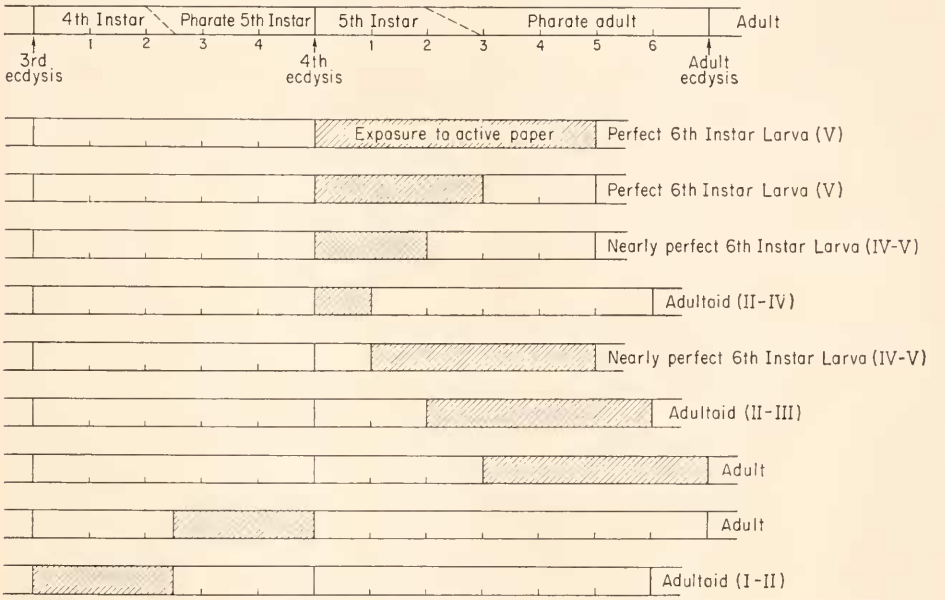

FIGURE 1. The top diagram illustrates the normal timing of developmental events when fourth instar *Pyrrhocoris* larvae are cultured at 25° C in the absence of PF; as illustrated by the two slanted, broken lines, the pharate stage of the succeeding instars begins two to three days after the third or fourth ecdyses.

The other nine diagrams illustrate the effects of "pulsed" exposure to active towelling during the periods indicated by cross-hatching; the Roman numerals refer to the intensities of the juvenile reactions as scored by the characters noted in Table I. For further details, see text.

in the absence of PF, this time is five days for each of the first four instars and seven days for the final (fifth) instar.

As pointed out previously (Sláma, 1964), the epidermal cells, at a certain stage in each instar, detach from the cuticle and retract to form an intervening space which is simultaneously flooded with moulting fluid (Passonneau and Williams, 1954). As Hinton (1958) has emphasized, these happenings signal the beginning of the "pharate" phase of the next instar. In the case of *Pyrrhocoris* larvae cultured at 25° C, the retraction of the epidermis is first initiated in the wing lobes about 48 hours after the preceding ecdysis; the retraction then spreads throughout the body

and finally reaches the epidermal cells of the abdomen 1½ to 2 days later. This timing of events is illustrated in the top diagram in Figure 1.

3. *Changes in the sensitivity to PF*

Five hundred larvae were hatched from eggs and subdivided into two equal lots. The control group was reared in contact with inert filter paper; the experimental group, in contact with towelling.

We would emphasize that, during the first four instars, the two groups were indistinguishable. Moulting occurred at five-day intervals and growth proceeded in a completely normal manner. But, at the conclusion of the fifth instar, a conspicuous difference was evident: the control group moulted into normal adults after the customary seven days, whereas all larvae exposed to PF moulted into perfect sixth instar larvae after five days.

This shows that PF is without any immediate influence on larvae possessing active corpora allata and high levels of endogenous juvenile hormone. Sensitivity first becomes evident in the fifth instar—that is, after the animal's own corpora allata have been inactivated and the secretion of juvenile hormone has stopped.

4. *Sensitivity of fifth instar larvae*

The sensitivity of final stage larvae was studied in further detail by placing groups of 6 or 7 individuals in contact with towelling for specific periods. The results, summarized in Figure 1, show that exposure throughout the first three days of the fifth instar was fully effective in blocking metamorphosis and provoking the formation of perfect sixth instar larvae. Exposure begun after the third day was completely ineffective.

Other experiments, summarized in Figure 1, document a declining sensitivity of *Pyrrhocoris* during the first three days of the fifth instar. Sensitivity is maximal during the first day; indeed, it was possible to show that freshly moulted individuals undergo abnormal metamorphosis as a consequence of only one hour of contact with filter paper impregnated with 5 mg PF.

5. *Sensitivity of fourth instar larvae*

As mentioned above, fourth stage larvae are seemingly unaffected by contact with PF. This finding was examined in the experiments diagrammed in the bottom two lines of Figure 1. One group of seven individuals was exposed to towelling during only the first half of the fourth instar; the other group, during only the second half.

As illustrated in Figure 1, exposure during the first half of the fourth instar provoked abnormal metamorphosis at the end of the fifth larval instar. By contrast, exposure limited to the second half of the fourth instar was inconsequential. This surprising result is discussed below.

6. *Stability of PF within the insect*

In testing the stability of PF, we took advantage of the prolongation of development which one can enforce by depriving the larvae of food. Thus, by starving

newly moulted larvae, the fifth instar can be prolonged by a corresponding number of days.

In the experiments summarized in Figure 2, four groups of seven individuals received a topical application of 10 μg of PF at the outset of the fifth instar. The groups were then starved for 1, 3, 6, and 10 days, respectively, before being fed.

As diagrammed in Figure 2, PF was sufficiently stable within the insect to permit a three-day prolongation of the fifth instar without any curtailing of the biological effects of the original topical application. When the period of starvation was extended to six days, or to ten days, the effects were progressively diminished. These findings show that PF is slowly metabolized and only gradually inactivated after its entry into the insect.

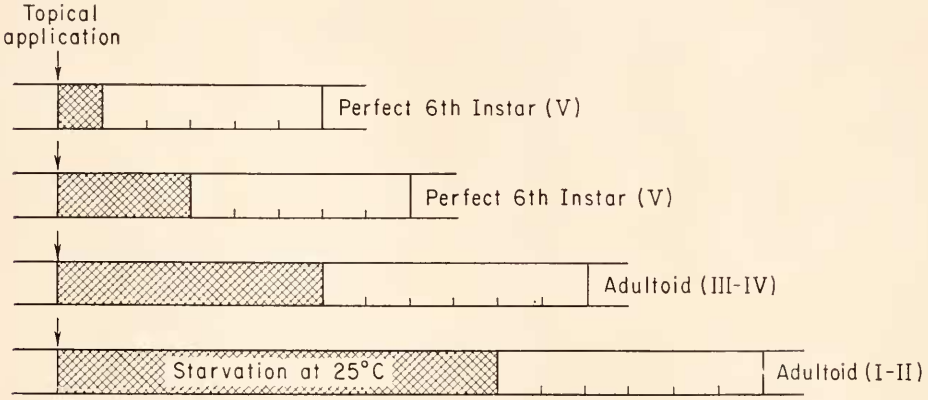

FIGURE 2. The effects of a single topical application of 10 μg of PF extract administered to *Pyrrhocoris* at the outset of the fifth larval stage. In order to prolong the instar, the four groups were starved at 25° C for one to ten days (as indicated by the cross-hatching). The findings document the slow inactivation of PF after its entry into the insect.

DISCUSSION

1. *Juvenile hormone in relation to larval growth and moulting*

Previous studies of *Pyrrhocoris* provide a frame-of-reference for interpreting the juvenile hormone activity of PF. For the most part, these previous investigations have been carried out by investigators in the Department of Insect Physiology in Prague.

By the extirpation, transplantation, and biological assay of living corpora allata, the corpora allata were shown to be active in the secretion of juvenile hormone throughout the first four larval instars (Sláma, 1962, 1964, 1965; Sláma and Hrubešová, 1963). Moreover, during this same 20-day period, the immature larvae were unaffected by the implantation of additional active corpora allata (Novák, personal communication; Sláma, unpublished observations).

In the present study, this result has been duplicated in the finding that continuous exposure to PF is without any detectable effects on growth and moulting during the first four larval instars. Evidently, as long as the titer of endogenous juvenile hormone remains above a certain level, the receptor mechanism at the cellular

level is "saturated" and, consequently, insensitive to the introduction of additional hormone.

2. *The critical period during the fifth instar*

Sláma (1964) has demonstrated that the corpora allata become inactive during the moult to the final (fifth) instar. If juvenile hormone is supplied by the implantation of active corpora allata, one can provoke the formation of a supernumerary sixth larval stage provided that the glands are implanted early in the fifth instar. Sensitivity is maximal during the first day after ecdysis and rapidly declines to zero during the first three days (Sláma, 1964). Manifestly, this is exactly the same result as that observed in the present study.

As summarized in Figure 1, we have been able to investigate the changing sensitivity to juvenile hormone with a resolution difficult to achieve by surgical manipulations. Thus, when the exposure of fifth instar larvae to active paper was "pulsed" for periods ranging from one hour to several days, the results were as follows: high sensitivity was documented for each of the first two days, less sensitivity during the third day, and zero sensitivity thereafter. In each region of the body, the loss of sensitivity to PF was synchronized with the activation of the epidermal cells as signaled by their detachment and retraction from the old larval cuticle. These findings are in full accord with those obtained by the implantation of active corpora allata (Sláma, 1964).

3. *Significance of the pharate condition*

As noted in the lowermost diagram in Figure 1, abnormal metamorphosis at the end of the fifth instar was provoked by contact with active paper during the first half of the fourth instar. This documents a remarkable stability of the paper factor once it has entered the insect.

It is therefore paradoxical to find that topical contact with PF was ineffective during the second half of the fourth instar (see next-to-bottom diagram in Figure 2). This result becomes intelligible when one recalls that the larvae are in the pharate condition during the final half of the fourth instar (see topmost diagram in Figure 1). Therefore, in order to gain entry into the body, PF would have to traverse, not only the cuticle and underlying epidermis, but also the intervening layer of aqueous moulting fluid.

Evidently, pharate larvae are resistant to the uptake of PF. This interpretation was tested by *injecting* PF into pharate fourth instar larvae. Of ten individuals subjected to this treatment, all reacted to PF by moulting into adultoid forms at the end of the fifth instar.

4. *Paper factor versus juvenile hormone*

As a physiological analogue of the juvenile hormone of *Pyrrhocoris,* PF appears to be more stable within the insect than is the native hormone itself. In addition to the evidence cited in the preceding section, we have observed that a single topical application at the outset of the fifth larval instar is effective in blocking normal metamorphosis, even when the next moult is postponed for many days

(Fig. 2). By virtue of this stability within the insect, PF as a juvenile hormone analogue shows more long-lasting effects than the native hormone, itself.

Summary

1. American paper products contain an extractable, heat-stable lipid (PF) with high juvenile hormone activity for the bug, *Pyrrhocoris apterus*. When reared in contact with "active paper" or extracts prepared from active paper, larval *Pyrrhocoris* undergo one or more supernumerary larval moults and finally die without completing metamorphosis or attaining sexual maturity.

2. As a potent analogue of the juvenile hormone of *Pyrrhocoris*, PF blocks those aspects of development which can take only in the absence of juvenile hormone—namely, the transformation of the fifth stage larva into an adult. By contrast, it fails to interfere in any way with normal growth and moulting during the first four larval instars—*i.e.*, with developmental events which normally take place in the presence of high levels of endogenous juvenile hormone.

3. Sensitivity to PF is maximal at the outset of the final (fifth) larval instar. At this particular stage, metamorphosis is blocked by contact with PF for a period as brief as one hour. High sensitivity persists during the first two days of the fifth instar and then declines to zero after the third day. In each region of the body, the loss of sensitivity to PF is synchronized with the activation of the epidermis, as signaled by its detachment and retraction from the old larval cuticle.

4. Several lines of evidence suggest that, after its entry into the larval insect, the paper factor is more stable, and therefore more long-lasting in its effects, than is the authentic juvenile hormone of *Pyrrhocoris*.

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